

Effects of Chronic Adolescent Fluoxetine Exposure on Adult Cognitive Function

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Abstract:

Adolescent-onset depression is becoming increasingly common in America. Many American teenagers take selective serotonin reuptake inhibitors (SSRIs) to treat the symptoms. Adolescence is, however, a period of critical brain development. Pharmacological intervention at such a time can lead to long term changes in adult cognition, yet literature is scarce on the effects of chronic adolescent SSRI use in particular. Our lab has found that the transcription factor Npas4 plays a role in the dramatic changes in the inhibitory system during adolescence, and that deficiencies in Npas4 during this period lead to long term cognitive deficits. It has also been shown that antidepressants can alter Npas4 expression in adult rats. Additionally, Npas4 mediates expression of the neurotrophic protein BDNF. BDNF is known to be upregulated in response to SSRI treatment, and overexpression of BDNF in wild type rodents can lead to learning and memory deficits. These findings suggest that Npas4 could mediate the effects of adolescent SSRI exposure on cognitive functions potentially by altering maturational processes that affect the inhibitory system during adolescence. As yet, no study exists that tracks the effects of chronic adolescent SSRI administration on Npas4 expression and its potential contribution to behavioral and cognitive modifications. To address this gap in knowledge, we compared the performance of wildtype C57Bl/6 mice treated with the SSRI fluoxetine in adolescence and adulthood in the Y-maze, Puzzle box, and Temporal order recognition memory (TORM) tests. These tasks are designed to measure prefrontal cortex-directed cognitive function. No changes in the cognition of mice treated with fluoxetine in adolescence were found in the Y-maze or Puzzle box. We observed a decrease in exploratory behavior in the learning phases of the TORM test in mice who had undergone treatment or vehicle protocol in adulthood, but no changes in object discrimination. No changes in BDNF expression were found across groups, but Npas4

expression in mice treated with fluoxetine in adulthood was significantly decreased compared to those treated in adolescence.

Table of Contents

Figures	
1. TORM Task	11
2. Puzzle Box Apparatus	12
3. Y-maze Results	16
4. TORM Results	17
5. Puzzle Box Results	18
6. Weight Gain Through Treatment	19
7. Western Blot Results	20
8. Puzzle Box Thigmotaxis	22
Table	
Table A	14
Introduction	5
Purpose	7
Materials and Methods	8
Animals	8
Fluoxetine Treatment	9
Behavioral Testing	9
Y-maze	9
TORM Test	10
Puzzle Box Test	12
Western Blot	14
Statistical Analysis	15
Results	16
Y-maze Results	16
TORM Results	17
Puzzle Box Results	18
Weight Gain During Treatment	19
Western blot results	20
Discussion	21
Conclusion	24
Recommendations	25
Acknowledgements	26
References	26

Introduction:

Teenage angst is often thought of as an inevitable byproduct of adolescence. We expect teens to be sad, and age out of their melancholy as they reach adulthood. But adolescence is a hotbed for the emergence of clinically diagnosable major depression (Thapar et al, 2012). As Susanna Schrobsdorff poignantly puts it in her TIME article about adolescent depression and anxiety: “the kids are not alright” (2016). According to Mojtabai et al., this trend has only been getting worse since 2005 (2016). In 2016, the NIMH estimated that 12.8% of American teenagers aged 12 to 17 had experienced at least one major depressive episode, and 70% of those individuals experienced severe impairment (“Major Depression”, 2017).

Antidepressants, common among which are selective serotonin reuptake inhibitors (SSRIs), are frequently prescribed to alleviate the symptoms of adolescent depression (Avenevoli et al., 2015). SSRIs work by inhibiting serotonin reuptake into the presynaptic terminal, thereby increasing availability of serotonin in the synapse (Hyttel, 1994). Though the fields of neuroscience and psychology have largely moved on from the 50-year old “serotonin hypothesis” of depression in favor of more complex, indirect models, SSRIs are still being prescribed because they are effective in treating certain subsets of depressed individuals (Albert et al., 2012). But more research must be done to characterize the long-term effects of SSRI administration on adolescent populations, because it has been consistently shown that adolescence is a period of significant reorganization in the prefrontal cortex (PFC) (Giedd et al., 1999; Giedd, 2004). During this time, the PFC is highly plastic and especially sensitive to environmental inputs (McEwan & Morrison, 2013).

Fluoxetine, brand name Prozac, used to be the only SSRI FDA approved for treating depression in children and adolescents (“Antidepressant Medications,” 2017; U. S. Food and

Drug Admin., 2007). Of the SSRIs approved today, fluoxetine has consistently been supported with the most evidence of effectiveness (Garland, 2016). Most studies regarding fluoxetine in adolescent rodents focus on mood-related behavior and the limbic system, with few discussing cognition. Sass and Wortwein did find that subchronic fluoxetine treatment of adolescent rats slightly impairs working memory and object recognition memory in adulthood (2012), but the mechanisms for these effects are unknown.

Our lab has been specifically interested in how the GABAergic system mediates such cognitive deficits. Adolescence is a critical period in GABAergic maturation (Klib, 2011) especially local GABAergic action (Thomas et al., 2013; Cass et al., 2013). There is much evidence that impaired GABAergic functioning can lead to deficits in PFC-directed cognitive function (Volk & Lewis, 2002). One protein that plays a key role in modulating inhibitory action and the excitatory/inhibitory balance is the transcription factor Npas4 (Coutellier et al., 2012). Our lab has recently found that Npas4 plays a pivotal role in refining prefrontal inhibitory circuits during the adolescence-adulthood transition. Deficiencies of Npas4 in adolescence, when it is normally highly expressed in the PFC, lead to PFC-dependent cognitive impairments that are sustained into adulthood in mice (Shepard et al., 2017).

There are no studies regarding the long-term effects of SSRIs on Npas4 in adolescent, wild type rodents. Guidotti et al found little difference in antidepressant mediated adult Npas4 expression in the PFCs of wild-type rats, but this study did not focus on adolescence (2012). Additionally, Maya-Vetencourt et al. did find increased Npas4 expression in the visual cortices of adult wild type rats exposed to chronic fluoxetine treatment (2012). Based on these results, Maya-Vetencourt proposed that serotonin signaling pathways activated by fluoxetine

administration lead to epigenetic changes in the Npas4 promoter region that enhance transcription (2013).

Interestingly, Npas4 is a molecular mediator of brain-derived neurotrophic factor (BDNF) (Lin et al., 2008), a neurotrophic protein implicated in, among other things, neuronal survival and synaptic plasticity (Martinowich & Lu, 2008). BDNF shows increased expression patterns in the hippocampus and prefrontal cortex following chronic SSRI treatment in adolescents and adults (Martinowich & Lu, 2008; Dincheva et al., 2017). While much focus has been placed on the positive cognitive correlates of upregulated BDNF in depressed patients (Homberg et al., 2014), there is also evidence that induced overexpression of BDNF can lead to impairments in learning and memory (Papaleo, 2011; Cunha et al., 2009).

We hypothesized that chronic adolescent fluoxetine treatment would lead to PFC-dependent cognitive deficits correlated with an upregulation of Npas4 and BDNF in the PFC.

Purpose:

Despite the increasing use of SSRIs in adolescent populations, their behavioral and molecular effects on cognition remain largely unknown. The drastic reorganization and vulnerability of the PFC in adolescence necessitates an analysis of the long term implications of adolescent SSRI use. This study sought to elucidate the effects of chronic adolescent SSRI exposure on adult PFC-directed cognitive function as well as a potential molecular mediator of these effects. Our findings will be able to help guide medical professionals on the long term safety of adolescent SSRI use.

Materials and Methods:

Animals:

32 male C57BI/6 (B6) mice were ordered from the Jackson Laboratory (Maine, US) and arrived at postnatal day (PND) 27. Mice were separated into 4 groups of n=8 mice per group in order to assess long term cognitive effects of fluoxetine exposure in adolescence. These groups followed a 2x2 model based on two variables: drug treatment (fluoxetine vs. vehicle) and age at treatment protocol (adolescent vs. adult).

Regarding drug treatment, mice were either delivered fluoxetine solution (10 mg/kg/day) or vehicle (water) through cage bottles. With regard to age, mice underwent treatment/vehicle protocol either during adolescence (PND 34-48) or during adulthood (PND 64-78). All groups underwent behavioral testing during adulthood. Henceforth, the 2 groups who were treated or provided with vehicle during adolescence will be referred to as the adolescent group. The 2 groups who were treated or provided with vehicle during adulthood will be referred to as the adult group.

The 2 adolescent groups were given 7 days to habituate to the colony room before undergoing the treatment procedure. During this period mice were group housed (4 mice per cage) with *ad libitum* access to food and water. During all steps of the experiment the colony room was kept on a 12 hour reverse light-dark cycle. The 2 adult groups were housed in the same colony room under the same conditions and began treatment at PND 64, when they had grown into adulthood.

Fluoxetine treatment:

The 2 adolescent groups underwent treatment either with fluoxetine or vehicle (water) from PND 34-48. The 2 adult groups underwent fluoxetine or vehicle treatment from PND 64-78. The fluoxetine group received 10 mg/kg/day of fluoxetine orally in drinking water from water bottles. Average water consumption relative to cage body weight was calculated prior to treatment in order to provide *ad libitum* water access throughout the treatment period. Water consumption and cage body weight was calculated every 2 days, and fluoxetine amount was adjusted based on these numbers. The vehicle groups were given *ad libitum* access to drinking water in water bottles and underwent the same procedures of body weight and water consumption measurement.

Behavioral testing:

Testing procedures began 14 days following the end of treatment to ensure fluoxetine washout. For 3 days prior to testing procedures, mice were handled once a day for one minute each. All testing was carried out during the dark phase of the light cycle.

Y-maze test:

Behavioral testing began with the Y-maze. The Y-maze is a one-day test used to measure spatial working memory and exploratory behavior through spontaneous alternation. Before testing began, mice were habituated to the testing room for 1 hour under red light. Testing procedures were run under red light as well. The Y-maze itself is a plastic apparatus composed of 3 arms in the shape of a “Y”. Mice were placed in the vertical arm to start and allowed to

explore the apparatus for 5 minutes. The apparatus was wiped down with ethanol prior to each mouse to eliminate scent cues.

Testing procedures were videotaped and the subsequent videos were scored based on 4 measures: total arm entries, number of triads, number of alternations, and percent alternations. One entry was defined as when all 4 paws of the mouse entered a new arm. The first entry was not counted in the total entries. Number of triads was defined as (Total number of entries – 2). An alternation was defined as each set of three consecutive different arm choices (arms ABC, BCA, CAB, etc). For example, if a mouse entered arm A, then arm B, then arm C, then arm A, this would count as 2 alternations. The percent of alternations was defined as

$$\left(\frac{\text{number of alternations}}{\text{number of triads}} \right) \times 100.$$

Temporal Order Recognition Memory (TORM) Test:

The TORM test assesses short term recognition memory. For 2 days prior to testing mice were habituated to the arena, a Pyrex glass square (40 cm x 40 cm) with a grey base. Before each arena habituation period and subsequent testing day, mice were habituated to the testing room for 1 hour under red light. The arena was wiped down with ethanol before each new mouse entered to eliminate scent cues. During habituation, mice were allowed to explore the arena for 15 minutes under red light. The same procedure was repeated on each of the two days.

Day 3 consisted of two sample phases (S1 and S2) and one testing phase (T), conducted under red light. These phases made use of two types of objects, clear bottles filled with water and stacked test tubes weighed down with coins. Two identical copies of one of the objects would be placed in the arena during S1, and mice were allowed to explore the S1 objects for a total of 4

minutes. A 30 minute delay followed between S1 and S2. During S2, two identical copies of the unused object would be placed in the same position in the arena as in S1, and mice were allowed to explore the S2 objects for 4 minutes as well. A 1 hour delay followed between S2 and T. During T, a new copy of the object from S1 and a new copy of the object from S2 were placed in the arena. Mice were allowed to the objects for a total of 3 minutes.

Half of each group of mice were assigned the bottles for S1, and the rest were given the tubes for S1. Half of each group were assigned the left position for the S1 object and the right position for the S2 object in the testing phase, and for the rest the opposite arrangement was used.

Each session was videotaped, and the total time sniffing each object was scored for the sample phases and the testing phases. A discrimination ratio was calculated for the testing phase and defined as $\frac{(\text{Time sniffing S1 object} - \text{Time sniffing S2 object})}{(\text{Time sniffing S1 object} + \text{Time sniffing S2 object})}$. Higher preference for the S1 object is correlated with more acute recognition memory.

Temporal order

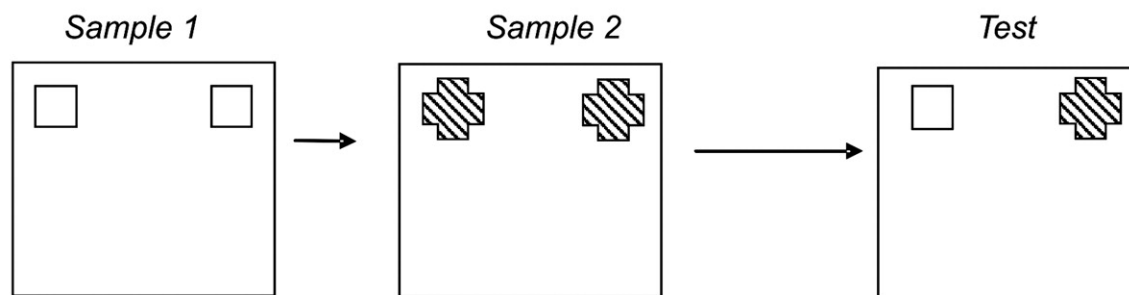


Figure 1: Illustration of the TORM Test. The white square and the striped cross each represent a different object. From (Barker et al., 2007).

Puzzle Box Test:

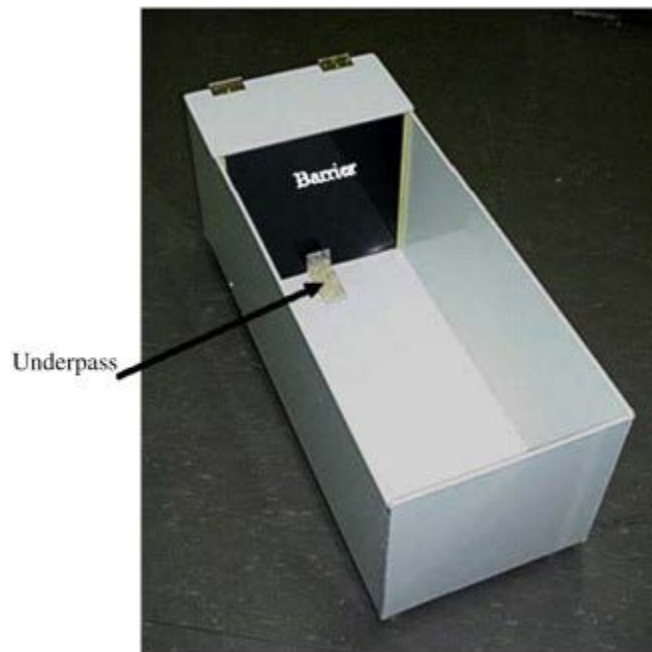


Figure 2: Example of a Puzzle box arena. Mice are assessed by their latency to enter the goal box through the underpass. From (Abdallah et al., 2001).

The puzzle box is a multi-purpose procedure that tests problem solving ability, learning and short term memory, and long term memory. Testing procedures begin with a 3 day period of food deprivation and habituation (Day 1-3) to food reward (Froot Loops) in order to make mice more responsive to the food reward during the test. Mice were restricted to 1 gram of food per day and $\frac{1}{4}$ of a Froot Loop. Their body weight was measured each day to ensure at least 90% body weight. All food was taken away 20 hours prior to the start of testing.

The arena is a white Plexiglas box separated into an uncovered start zone (58 cm x 28 cm) and a covered goal zone (15 cm x 28 cm) by a barrier containing a small underpass (2 cm x 4 cm x 4 cm).

Testing was divided into 9 trials that took place over 3 days. 3 trials were conducted per day. All trials were conducted under dim fluorescent light to provide a light incentive to enter the goal box. Mice were habituated to the testing room in the dark for 1 hour prior to testing

procedures on each day. During the trials, mice were placed at the far end of the start box and their latency to enter the goal box was recorded by reviewing video. Mice were allowed to remain in the arena for 2 minutes following goal box entry before the next trial began. The arena was wiped down with ethanol before each new mouse.

During Day 4, mice were trained to enter the goal box. In T1-3, a food reward was placed in the goal box, and the underpass was unblocked. *Ad libitum* access to food was restored at the end of Day 4. No food reward was provided for any trial following T3. On Day 5, mice underwent T4-6. T4 was identical to T3, minus a food reward. In T5 and T6, the underpass was filled with sawdust. Mice would have to dig through the sawdust to enter the goal box. On Day 6, mice underwent T7-T9. T7 was identical to T6. In T8 and T9, the underpass was obstructed with a cardboard plug (2 g, 2.5 cm x 7.5 cm x 0.5 cm cardboard piece attached to a cardboard block of 3 cm x 2 cm x 1.5 cm). Mice would have to use their teeth, paws, or body to physically move the plug out of the way to enter the goal box.

Problem solving ability was measured through T5 and T8, when mice were first introduced to new obstacles. T3, T6, and T9 tested short term memory as mice had to recall how to enter the goal box under certain conditions. T4 and T7, wherein the mice were prompted to recall how to enter the box based on learning that occurred 24 hours prior, indicated long term memory.

Day	Day 1	Day 1	Day 1	Day 2	Day 2	Day 2	Day 3	Day 3	Day 3
Trial	1	2	3	4	5	6	7	8	9
Underpass	Open	Open	Open	Open	Sawdust	Sawdust	Sawdust	Plug	Plug
Function	(Training)	(Training)	L/STM	LTM	PS	L/STM	LTM	PS	L/STM

Table A: Puzzle box trials by day, status of the underpass, and cognitive function measured. L: Learning/STM: Short term memory/LTM: Long term memory/PS: Problem solving.

Western Blot:

Mice were euthanized 90 minutes following the end of the last Puzzle box trial. Brains were rapidly extracted and flash frozen for storage at -80°C . PFCs were dissected out in a -20°C cryostat and placed on dry ice for transport. PFC samples were stored at -80°C .

Western blot analysis was carried out to determine protein levels of Npas4 and BDNF in the left PFC with $n=3-4$ per group. Tissue was homogenized on ice with lysate buffer (2.5 mL T-PER Tissue Protein Extraction Reagent, $\frac{1}{4}$ Protein Inhibitor tablet) at a volume determined by tissue weight. Samples were centrifuged (10,000G, 30 min, 4°C) and the supernatant was taken. 10 μL of supernatant were taken for a Bradford Protein Concentration Assay in order to determine amount of sample to load into the Western Blot gel. Supernatant was diluted (1 μL sample, 19 μL ddH₂O) and plated in triplicate on a microplate next to albumin standards (Thermo Fisher Scientific) and blanks (ddH₂O). Coomassie Plus (Thermo Scientific) was added to each well, and the samples were analyzed with ChromaMate Reader software.

100 μL supernatant was mixed with 25 μL dithiothreitol and 125 μL sample buffer (2.5 mL Tris HCl pH 6.8, 5 mL glycerol, 4 mL 10% SDS, 2 mL 0.1% bromophenol blue, 5.5 mL ddH₂O) and stored at -20°C . Samples were boiled at 95°C for 3 minutes before being loaded

into Novex Wedgewell 14% Tris-Glycine gels (Thermo Fisher Scientific) and run through gel electrophoresis (Invitrogen Novex XCell SureLock Mini-Cell) in running buffer (3 g Tris, 14.4 g glycine, 1 g SDS). Gel was transferred to membrane (LI-COR) soaked in transfer buffer (200 mL 25x TB, 100 mL methanol, 700 mL ddH₂O). Once transferred, membrane was washed for 5 minutes in PBS on a shaker, then washed for 1 hour in blocking buffer (50 mL 1x TBST, 2.5 g nonfat dry milk) on a shaker. Following this, membrane was incubated with diluted primary antibody (1 μ Ab) for Npas4 (Harvard) (1:2000), BDNF (Santa Cruz Biotechnology) (1:400), and Beta-actin (B-actin) (Abcam) (1:1000) as a reference gene overnight at 4 $^{\circ}$ C.

The next day, membrane was washed 3 times with TBST for 5 minutes each. Secondary antibody (2 μ Ab, IRDye 800CW Goat anti-rabbit) (1:10,000) was added and membrane was incubated on the shaker while covered at room temperature for 1 hour. Membrane was then washed with wash buffer 3 times for 5 minutes each on the shaker while covered. Membranes were developed using LI-COR Biosciences Odyssey CLx Imaging System and analyzed using LI-COR Image Studio. BDNF levels were measured via proBDNF precursor. All Npas4/proBDNF data were normalized to B-actin.

Statistical Analysis:

Data were analyzed in the program GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, USA) using 2 by 2 analysis of variance (ANOVA) tests where treatment and age-at-treatment were the independent variables. When significant main effects were found, *post-hoc* Tukey's multiple comparisons test was used to compare group means. All results are shown with mean and standard error of the mean (SEM).

Results

Y-maze:

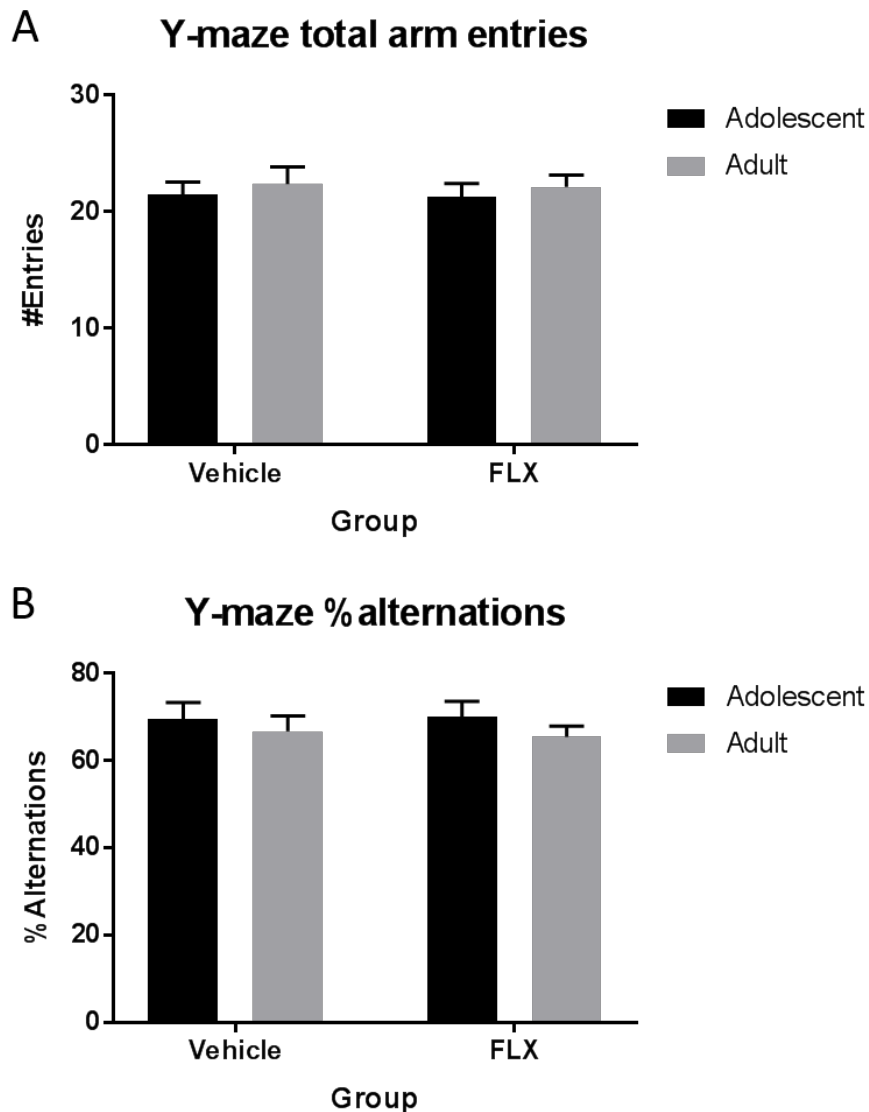


Figure 3: No significant treatment or age-at-treatment effects were found across number of arm entries (Fig. 3A) or percent alternations (Fig. 3B) in the Y-maze.

Mice treated with vehicle (control) or fluoxetine (treatment) in either adolescence or adulthood were tested in adulthood in the Y-maze. No significant treatment or age-at-treatment effects were found across number of arm entries (Fig. 3A) or percent alternations (Fig. 3B).

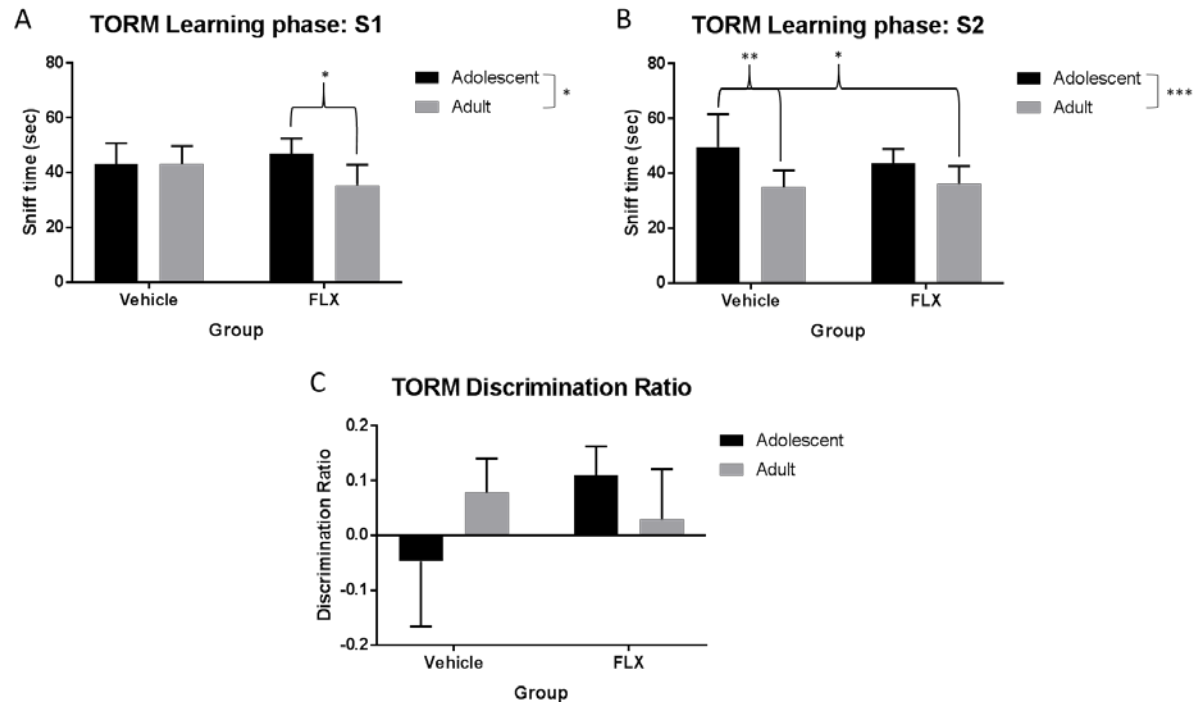
TORM Test:

Figure 4: A significant age-at-treatment effect ($p=0.0308$) and age-at-treatment*treatment interaction ($p=0.0297$) were found during the S1 learning phase (Fig. 4A). A significant age-at-treatment effect was found during the S2 learning phase ($p=0.0007$) (Fig. 4B). No significant differences across groups were found in the discrimination ratio (Fig. 4C).

2-way ANOVA analysis of data from the TORM test revealed a significant age-at-treatment effect ($p=0.0308$) and age-at-treatment*treatment interaction effect ($p=0.0297$) during the S1 learning phase (Fig. 4A). *Post-hoc* analysis revealed a significant difference between the adolescent FLX group and the adult FLX group ($p=0.0219$). We also found a significant age-at-treatment effect ($p=0.0007$) during the S2 learning phase (Fig. 4B). *Post-hoc* analysis showed significant differences between the adolescent vehicle group and the adult vehicle group ($p=0.0054$) and between the adolescent vehicle group and the adult FLX group ($p=0.0147$). These results suggest that mice who enter treatment/vehicle protocol in adulthood spend less time exploring novel objects. However, no significant differences were found across groups with regards to discrimination ratio (Fig. 4C).

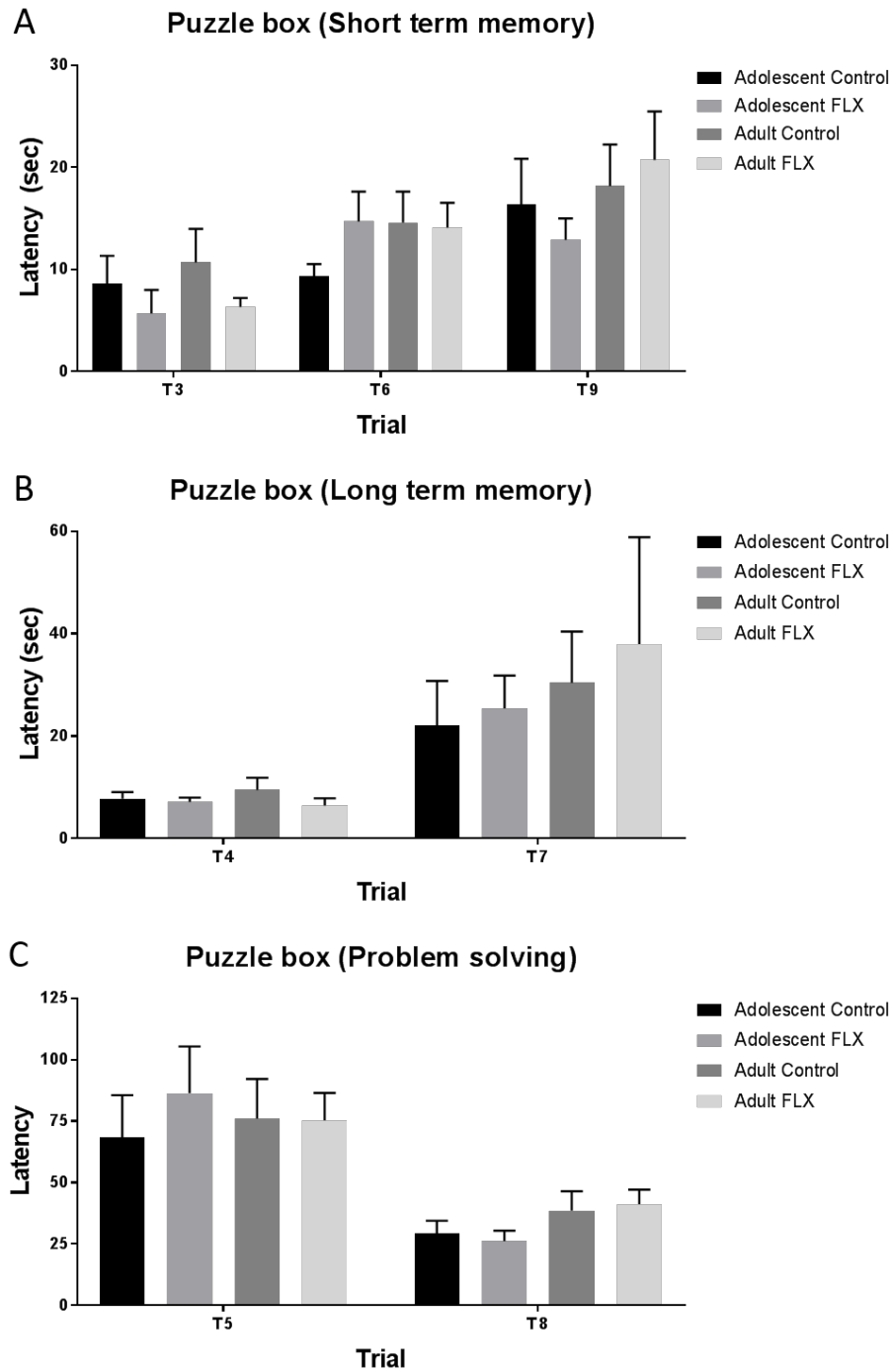
Puzzle box test:

Figure 5: No differences were found across groups in latency to enter the goal box across the 9 trials of the Puzzle box test. Trial data are presented grouped by cognitive function measured.

Latency to enter the goal box was measured in the Puzzle box test trials. Analysis revealed no significant treatment or age-at-treatment effects across groups and across cognitive function tested – short term memory (Fig. 5A), long term memory, (Fig. 5B), and problem solving (Fig. 5C).

Treatment weight gain:

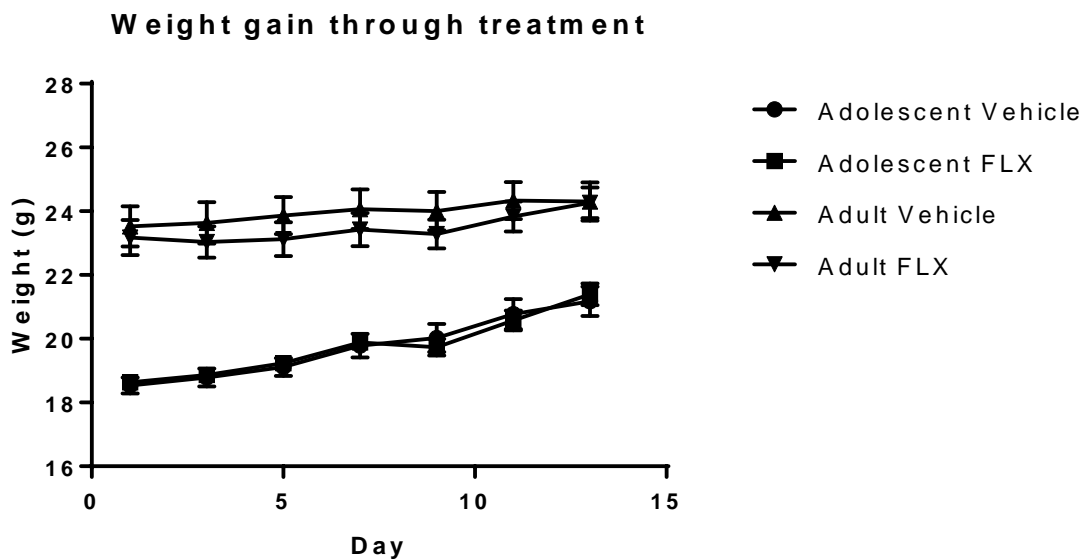


Figure 6: Weight gain was tracked in all groups throughout the drug and vehicle treatment protocol. As expected, rate of weight gain was significantly higher in the adolescent groups. No significant treatment effects were observed.

Body weight was tracked through drug and vehicle treatment (Fig. 6) in order to normalize fluoxetine treatment by cage weight. Rate of change of weight was significantly different between adolescent and adult groups ($p < 0.0001$), as is to be expected when comparing grown subjects and subjects which are still developing. No significant treatment effects were found.

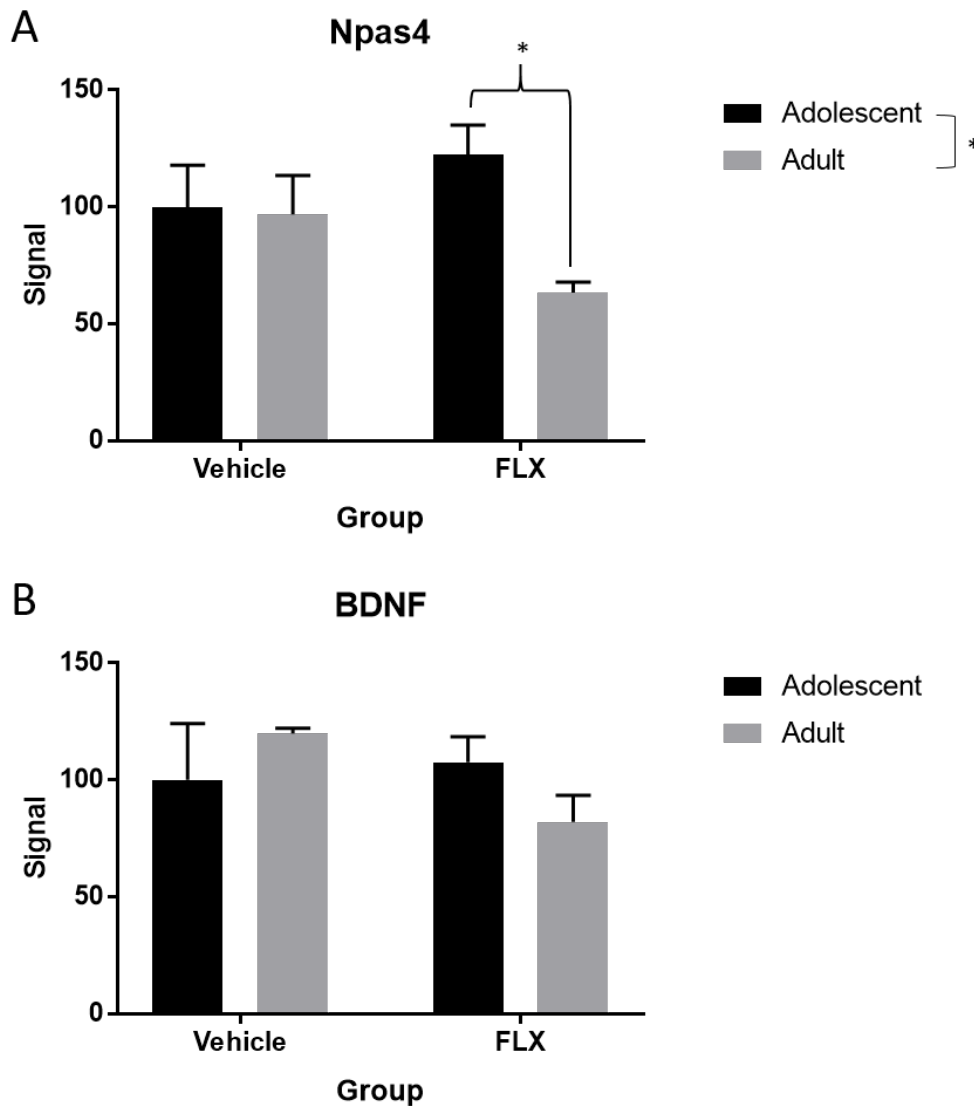
Western blot:

Figure 7: A significant reduction in Npas4 expression was found in the adult FLX group compared to the adolescent FLX group ($p=0.0474$). No differences were found across groups in proBDNF expression. All data were normalized to B-actin.

2-way ANOVA analysis of Npas4 expression in the PFC indicated a significant age effect ($p=0.0451$). *Post-hoc* analysis revealed that Npas4 expression was significantly reduced in the adult treatment group compared to the adolescent treatment group ($p=0.0474$) (Fig. 7A). No significant differences were found in proBDNF expression across groups (Fig. 7B).

Discussion

Present data show that in a non-depressed mouse model, chronic fluoxetine treatment both in adolescence and adulthood does not significantly affect the PFC-directed cognitive functions assayed. Npas4 expression in mice treated with fluoxetine in adulthood but not adolescence is significantly decreased, and BDNF levels are unchanged.

We found no significant treatment or age-at-treatment effects in either number of arm entries or percent alternations in the Y-maze, suggesting that adult spatial working memory is not significantly affected by adolescent fluoxetine treatment. Similarly, we found no significant differences between groups on measures of short term memory, long term memory, and problem solving in the Puzzle box test.

We did not find significant changes in the discrimination ratio between temporally separated objects in the TORM test across groups. These results echo Sass and Wortwein, who did not find differences in object recognition memory or object-in-place recognition memory in adult rats who had been treated with fluoxetine as adolescents (2012).

Age-at-treatment and age-at-treatment*treatment interaction effects in the learning phases of the TORM test showing that the adolescent group displayed increased exploratory behavior were unexpected, as all mice were tested in adulthood. Though the adult treatment/vehicle groups were approximately 1 month older than the adolescent treatment/vehicle groups during behavioral testing, both testing periods were contained within young adulthood. To test whether this may be indicative of anxiety behavior, we analyzed thigmotaxis (percentage of time spent near the walls of the arena) in the first trial of the Puzzle box. We found no significant differences among groups (Fig. 8), however further tests of anxiety would be needed to confirm.

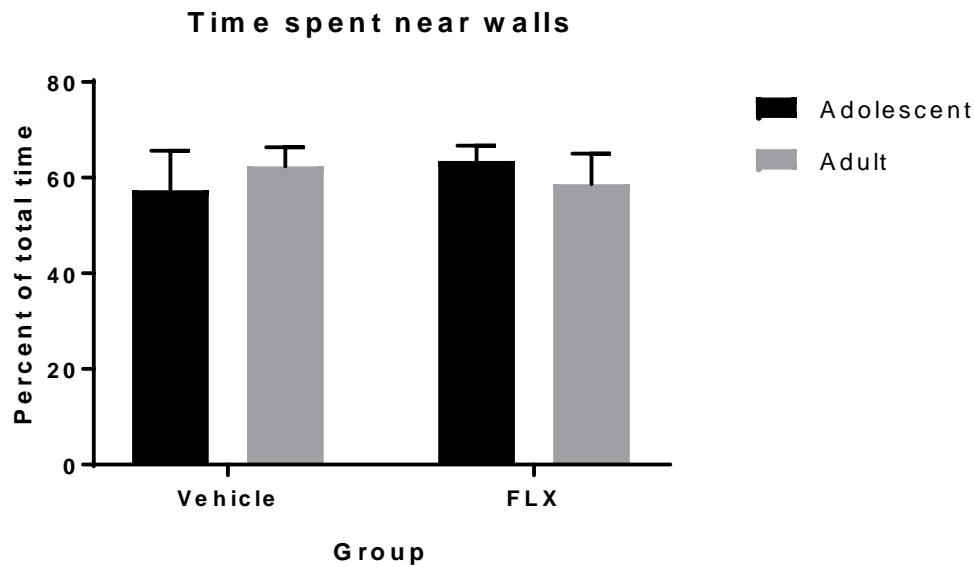


Figure 8: Thigmotaxis (time spent near walls) was calculated as a percentage of total time spent in the lighted section of the Puzzle box during the T1 trial. No significant differences were found across groups.

There are differing reports on how adolescent fluoxetine treatment itself affects adult anxiety behavior. Norcross et al. did not find significant adult anxiolytic or anxiogenic effects of adolescent fluoxetine treatment in B6 mice (2008) while Iñiguez et al. did find increased long term sensitivity to anxiogenic stimuli in rats, but no long term effects on object exploration in a familiar environment (2010).

Additionally, this TORM protocol was adapted from Barker et al. (2007), who studied temporal order recognition memory in rats. That the control mice displayed discrimination ratios that were not significantly different from chance level (“0”) indicates that the protocol may need to be adjusted even more, perhaps with shorter ITIs or longer learning periods.

While behavioral data indicated that chronic fluoxetine treatment in adolescence does not significantly affect cognitive function in adulthood, it is important to consider that this might be the result of compensatory mechanisms in the serotonergic system.

Our Western blot findings suggest that, contrary to expectation, adult but not adolescent fluoxetine treatment may cause long term decreases in Npas4 expression in the PFC. Guidotti et al. found little difference in Npas4 expression in the PFCs of adult duloxetine treated rats, but 2 important caveats should be made when comparing results (2012). First, duloxetine is a serotonin-norepinephrine reuptake inhibitor (SNRI), thus Npas4 results could also be affected by increased norepinephrine transmission. Secondly, Guidotti et al. collected brains 24 hours following the cessation of duloxetine treatment, meaning Npas4 expression was measured in the short-term post treatment.

Reduced expression of Npas4 in the adult fluoxetine group did not seem to affect PFC-directed cognitive function. These results are in line with previous findings from our lab that adult Npas4 heterozygous knockdown mice perform comparably to wild-type controls in an object/context mismatch test (Coutellier et al., 2015). Additionally, we have found that knockdown of Npas4 during the post-adolescent period does not lead to cognitive deficits (Shepard et al., 2017).

It is unclear from the present study whether Npas4 levels are lowered in adolescence and recover to baseline expression by adulthood, or if the adolescent brain is altogether protected against fluoxetine's effects on Npas4. To determine this, an adolescent treatment group killed shortly following treatment cessation should be added to determine short term effects of adolescent fluoxetine exposure on Npas4 expression.

We did not find significant treatment or age-at-treatment effects on proBDNF expression. Though we expected changes in Npas4 expression to correlate to changes in BDNF expression, BDNF expression is activity dependent and modulated by many other factors at each stage of synthesis and transport (Lu, 2003). Further investigation is required to determine which other

factors might attenuate the effects of decreased Npas4 expression. Furthermore, present studies have only reported on Npas4's effects on mature BDNF (mBDNF) expression levels. It is unlikely that Npas4's effects on the proBDNF precursor correlate exactly with its effects on the mature protein. Direct measurement of mBDNF would produce results that could be more readily compared to previous studies.

Conclusion

Chronic adolescent fluoxetine treatment does not seem to significantly affect PFC-directed cognitive function in adulthood. This lends support to the long term cognitive safety of fluoxetine use in adolescents. However, it is possible this is a result of compensatory mechanisms in response to SSRI challenge. Chronic fluoxetine treatment does seem to decrease Npas4 expression when administered in adulthood but not adolescence. ProBDNF levels, meanwhile, are left unaffected. It would be prudent to expand these procedures to both female and depressed models of both sexes, as well as to short term fluoxetine groups.

Recommendations

The most pertinent first step for future research would be the introduction of female subjects. In humans, females suffer from depression at a higher rate than males. However, care would have to be taken into selection of the time period of interest, as there is evidence that the PFC in females undergoes reorganization earlier than in males. Next, these procedures should be implemented in depressed models. This study serves as a baseline model, but the behavioral and genomic profiles of individuals with depression vary from their non-clinical counterparts both with and without SSRI treatment. Additionally, this study investigated mid-adolescence in male mice, but early adolescence may be an even more vulnerable time for PFC development. Again, periods of enhanced vulnerability may differ between male and female populations. Further behavioral tests could also be conducted to parse potential differences in attention. In order to more thoroughly assess the timeline of potential changes in *Npas4* expression in response to fluoxetine treatment, groups of mice killed shortly after treatment should be introduced. As well, more direct measures of mBDNF should be utilized to verify unchanging BDNF expression.

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